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Involvement of opioid system in behavioral despair induced by social isolation stress in mice

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ABSTRACT

Social isolation stress (SIS) as a type of chronic stress could induce depressive- and anxiety-like behaviors. Our study evaluates the role of opioid system on negative behavioral impacts of SIS in male NMRI mice. We investigated effects of morphine, a nonselective opioid receptor (OR) agonist, naltrexone (NLX), an OR antagonist, naltrindole (NLT), a delta opioid receptor (DOR) antagonist, SNC80, a DOR agonist, U-69593, a kappa opioid receptor (KOR) agonist, nor-Binaltorphimine, a selective KOR antagonist and cyprodime hydrochloride a selective mu opioid receptor (MOR) antagonist on depressive- and anxiety-like behaviors. Using RT-PCR we evaluated ORs gene expression in mice brain. Our findings showed that SIS induced anxiety- and depressive-like behavior in the forced swimming test, open field test, splash test and hole-board test. Moreover, administration of SNC-80 significantly mitigated anxiety- and depressive-like behaviors. NLT decreased grooming-activity in the splash test. Excitingly, administration of agents affecting KOR failed to alter the negative effects of SIS. RT-PCR demonstrated that MOR and KOR gene expression decreased in socially isolated mice; however, SIS did not affect DORs expression. Our findings suggest that SIS at least in part, probably via altering endogenous opioids particularly MORs and KORs but not DORs mediated negative impacts on behavior; also, it could be concluded that DORs might be considered as a novel target for studying depression and anxiety.

1. Introduction

Social isolation stress (SIS) is considered as a valid animal paradigm to study the effects of chronic early life stress on later behaviors [1–3]. Clinical and preclinical studies have shown that experiencing early-life stresses could profoundly affect mood and behavior in the adulthood [4–7]. Social isolation stress is a type of chronic stress that could increase seizure susceptibility, depressive- and anxiety-like behaviors [8–11]. Few molecular pathways have been proposed to be involved in these outcomes, although the exact mechanisms responsible for adverse effects of SIS are still unclear. Past studies have suggested that SIS could induce neurochemical changes in the brain [12–17]. However, the involving of opioid system in depression- and anxiety-like behavior following SIS is not clearly investigated.

It has been suggested that opioid system is involved in various pathways including pain, depression, anxiety, memory, addiction and seizure [18–22]. Previous studies have demonstrated that opioid system regulates the natural activities such as sexual and social behaviors [23–25]. Endogenous opioids interact with opioid receptors including mu- (MOR), delta (DOR) and kappa (KOR) -opioid receptors [26]. Recently, it has been determined that stress could impact on opioid system by increasing the levels of dynorphin as an endogenous KOR ligand [27]. Animal studies revealed that lack of opioid receptors induced depressive- and anxiogenic-like behaviors [28].

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The aim of present study is to investigate the role of opioid system in depression- and anxiety-like behaviors in a mouse model of SIS. For reaching this goal, we investigated the effect of morphine, cyprodime hydrochloride (CYP) naltrexone (NLX), naltrindole (NLT), U-69593, nor-binaltorphimine (nor-BNI), and SNC80 on depressive- and anxiety-like behaviors in socially isolated animals. Also, we evaluated the gene expression of each opioid receptor using RT-PCR method in the hippocampus and amygdala samples.

2. Materials and methods

2.1. Animals and housing conditions

Male NMRI mice aged 21–25 days and weighing 10–12 g were used. Animals were housed in two different conditions including social condition (SC) and isolated condition (IC). All animals were kept under standard laboratory conditions i.e. temperature: 22 ± 2 °C, humidity: 50 ± 10%, 12-h light–dark cycle, and ad-libitum access to food and water for a period of 5 weeks. Socially conditioned mice were placed in plexiglas boxes (25 cm × 25 cm × 15 cm) (6 mice per cage) and IC animals were placed individually in Plexiglas boxes (24 cm × 17 cm × 12 cm) [8,12]. In order to diminish handling and social interaction cages of IC animals were cleaned weekly by the same experimenter. All experiments were carried out between 10:00 a.m. and 02:00 p.m. Each mouse was used only once for each test. Each trial group contained 6–8 animals. All tests were performed in accordance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication #80-23) and institutional guidelines for animal care and use.

2.2. Drugs

Morphine sulfate (non-selective opioid receptor agonist, 1 and 5 mg/kg), Naltrexone or NLX (non-selective opioid receptors antagonist), (+)-4-(αR)-α-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl80-*N,N*-diethylbenzamide or SNC80 (selective DOR agonist), Naltrindole or NLT (selective DOR antagonist), U-69593 (selective KORs agonist), nor-Binaltorphimine (nor-BNI, selective KORs antagonist), Cyprodime hydrochloride (CYP, a selective MOR antagonist) and Pentylenetetrazole (PTZ) were purchased from Sigma (Sigma, St Louis, MO, USA).

2.3. Experiment design and treatments

In the first step, behavioral tests including forced swimming test (FST), open-field test (OFT) and the splash test were used to evaluate anxiety- and depressive-like behaviors in both social and isolated condition animals. On the next step, the possible effects of opioid receptor agonists/antagonists on socially isolated mice were assessed using above-mentioned tasks. In this regard, mice (IC and SC) were treated with morphine (1 and 5 mg/kg, i.p. 30 min prior to tests), naltrexone (3 mg/kg, i.p. 30 min prior to test), nor- nor-Binaltorphimine (5 mg/kg, i.p. 30 min prior to test) Cyprodime hydrochloride (3 and 10 mg/kg, i.p. 60 min prior to test), SNC80 (1 mg/kg, i.p. 60 min before tests), naltrindole (1 mg/kg, i.p. 30 min before tests), and U-69593 (3 mg/kg subcutaneously, 15 min prior to test). Naltrindole, morphine and naltrexone were dissolved in saline and injected intraperitoneally (i.p.) 30 min before the tests in a constant volume of 5 ml/kg body weight [29–34]. SNC80 and nor-Binaltorphimine was dissolved in dimethylsulfoxide (DMSO; 5%) and diluted in physiological saline solution [35,36]. Also, cyprodime hydrochloride was dissolved in 1% ethanol and U-69593 (3 mg/kg) was suspended in 25% propylene glycol [33,37,38]. To exclude the possible impact of solvents, DMSO, propylene glycol and saline were also used as vehicles at constant volume of 5 ml/kg. The doses and time of drug administrations were chosen based on our pilot study and also above-mentioned published reports. In the final step, after removing hippocampus and amygdala from the brain, we evaluated the gene expression of opioid receptors including MORs, KORs and DORs.

2.4. Open-field test (OFT)

Open-field test was done just before the FST to assess the locomotor activity of animals [39,40]. OFT was used to exclude the possibility that changes in immobility time are not the result of modifications in motor activity and also to interpret the locomotor activity in response to SIS [41]. The apparatus of OFT was made of white opaque plexiglas (50 cm × 50 cm × 30 cm) and was softly illuminated. Each mouse was placed gently on the central area (30 cm × 30 cm) and behaviors were documented by a camera in a 5 min period and were analyzed by Ethovision software version 8 (Noldus, Netherlands). The apparatus was cleaned with 70% ethanol after each experiment. The distance moved (horizontal activity) and the numbers of rearing (vertical activity) and time spent in central zone (central activity) were evaluated.

2.5. Forced swimming test (FST)

The FST was conducted based on previously described method [42–44]. Mice were separately placed in an open cylinder-shaped bottle (diameter 10 cm, height 25 cm), containing 19 cm water (23 ± 1 °C). Mice were permitted to swim for 6 min and the period of immobility was recorded during the last 4 min of the test. Each mouse was judged to be immobile when it terminated struggling and stayed floating immobile, making only those activities necessary to keep its head above the water.

2.6. Splash test

In order to evaluate the self-care and motivational behaviors splash test was performed. In this paradigm, grooming activity time, as an indirect measure of palatable solution intake, was evaluated. 10% sucrose solution was sprayed on the dorsal coat of animals. The total grooming activity time was recorded during 5 min after the sucrose vaporization [10,45]. Grooming activity consists of nose/face grooming, head washing and body grooming.

2.7. Hole board test (HBT)

Hole-board test is considered as a reliable trial to assess the anxiety-like behaviors in the rodents [46]. The apparatus was made of a white Plexiglas square (50 cm × 50 cm) with 16 equally sized holes (3 cm in diameter) and was located 50 cm above the floor. Mice were placed in the center of the board, and the number of head-dips was calculated in a 5-min period by an experimenter.

2.8. Real-time RT-PCR analysis for hippocampus opioid receptor genes

Hippocampus and amygdala were removed and immediately frozen on dry ice, and then stored at −80 °C. In the first step total RNA was extracted from hippocampi and amygdala using Trizol reagent (Invitrogen, Cergy Pontoise, France). Alterations in the mRNA levels of genes were determined using qRT-PCR after the reverse transcription of 1 μg of RNA from each sample using PrimeScript RT reagent kit (Takara Bio Inc., Otsu, Japan). qRT-PCR was completed on a light cycler device (Roche Diagnostics, Mannheim, Germany) using SYBR Premix Ex Taq technology (Takara Bio). Sequences of primers are shown in Table 1. Thermal cycling conditions included an initial activation step for 30 s at 95 °C afterwards 45 cycles as well as a denaturation step for 5 s at 95 °C and a combined annealing/extension step for 20 s at 60 °C. Melting curve analysis was performed to certify whether all primers yielded a single PCR product. Hypoxanthine phosphoribosyl transferase1 (Hprt1), was considered as a normalizer and fold changes in expression

Table 1
Primers for RT-PCR.

Gene name	Primers (Forward and reverse)
Opioid Receptor, mu 1	F: GAGCCACAGCCTGTGCCCT R: CGTGCTAGTGGCTAAGGCATC
Opioid Receptor, Kappa 1	F: CCTGGCATCATCTGTTGGTA R: GGAAACTGCAAGGAGCATTC
Opioid Receptor, Delta 1	F: GCTCGTCATGTTTGGCATC R: AAGTACTTGGCGCTCTGGAA
Hprt1	F:TGCTCGAGATGTGATGAAGG R: AAGCAGATGGCCACAGAACT

of each target mRNA relative to Hprt1 was calculated based on $2-\Delta\Delta Ct$ relative expression formula as described previously [6,47,48]

2.9. Statistical analysis

Comparisons between the groups were assessed using *t*-test and one-way ANOVA followed by Tukey's post hoc test using GraphPad Prism 7 software (San Diego, CA, USA). *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of SIS on depressive- and anxiety-like behaviors

Results obtained from the *t*-test analysis revealed that SIS significantly increased immobility time in the FST ($t = 10.17$, $df = 14$, $P < 0.001$, Fig. 1A) and also significantly decreased grooming activity time in the splash test ($t = 9.355$, $df = 14$, $P < 0.001$, Fig. 1B) in comparison with SC animals. In the OFT, IC animals showed higher locomotor activity, considering total distance moved ($t = 3.594$, $df = 14$, $P < 0.01$, Fig. 1C) and number of rearings ($t = 4.248$, $df = 14$, $P < 0.001$, Fig. 1D) compared to SC animals. Also, analysis showed that time spent in the central zone of OFT in socially isolated animals significantly decreased in comparison to normal condition ($t = 3.219$, $df = 10$, $P < 0.01$, Fig. 1E). *t*-test analysis on results of the HBT showed an anxiety-like behavior in IC mice ($t = 4.213$, $df = 14$, $P < 0.001$, Fig. 1FE) when compared to SC animals.

3.2. Effect of opioid receptors agonists and antagonists on depressive- and anxiety-like behaviors

In this step, we tried to investigate the role of opioid system in the pathophysiology of depressive- and anxiety-like behavior following SIS paradigm. One-way ANOVA analysis demonstrated that administration of opioid receptor agonists and antagonists could impact the immobility time in the FST ($F(9, 61) = 0.7709$, $P < 0.001$, Fig. 2A). Tukey's analysis showed that administration of higher dose of morphine (5 mg/kg, $P < 0.05$), U-69593 (3 mg/kg, $P < 0.01$), higher dose of CYP (10 mg/kg, $P < 0.05$), and nor-BNI (5 mg/kg, $P < 0.05$) in SC animals, significantly increased immobility time in the FST in comparison to vehicle-treated SC mice. On the other hand, administration of these drugs in IC animals showed that treatment with SNC80 significantly decreased immobility time when compared with vehicle-treated counterpart ($P < 0.001$, Fig. 2B).

In the splash test, one-way ANOVA analysis revealed that administration of opioid receptor agonists and antagonists could affect grooming activity time in both IC ($F(9, 70) = 2.59$, $P < 0.001$, Fig2C) and SC animals ($F(9, 70) = 1.277$, $P < 0.001$, Fig2D) compared to vehicle-treated counterparts. Tukey's analysis showed that administration of morphine at higher dose (5 mg/kg, $P < 0.01$) and U-69593 (3 mg/kg, $P < 0.05$) in SC mice, significantly decreased grooming time, however, injection of NLX at 3 mg/kg and CYP at 10 mg/kg increased the grooming time ($P < 0.05$) when compared to vehicle-treated animals. Also in IC animals, post Tukey's analysis showed that injection of NLT at 1 mg/kg significantly decreased ($P < 0.05$) and SNC80 (1 mg/kg) increased ($P < 0.01$) grooming activity time compared to vehicle-treated animals.

One-way ANOVA analysis showed that administration of morphine (1 and 3 mg/kg), NLX (3 mg/kg), NLT (1 mg/kg), and SNC80 (1 mg/kg) could not affect the distance moved at the OFT in both IC ($F(9, 70) = 1.02$, $P > 0.05$, Fig. 2F) and SC animals ($F(9, 70) = 1.257$, $P > 0.05$, Fig. 2E). In addition, one-way ANOVA failed to show any significant differences between administrations of these drugs in the number of rearings in both IC ($F(9, 70) = 0.3355$, $P > 0.05$, Fig2H) and SC mice ($F(9, 70) = 0.7794$, $P > 0.05$, Fig. 2G) when compared to vehicle-treat mice.

In the final step we evaluated the anxiety-like behavior in both IC and SC animals after administration of all the mentioned drugs in both HBT and OFT (time spent in the central zone). Result obtained from the one-way ANOVA analysis showed that none of the administrated drugs

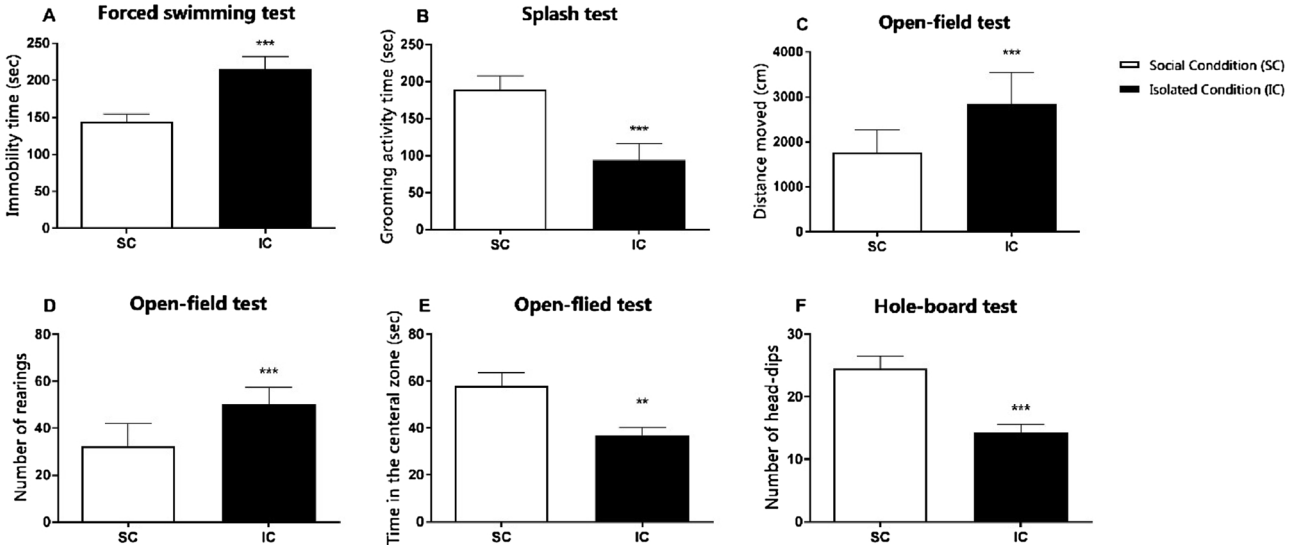

Fig. 1. Effects of social isolation stress (IC) on behavioral despair in immobility time of the FST (A), self-care behavior in the Splash test (B), locomotor activity in the OFT (C and D), and anxiety-like behavior in the OFT (time spent in central zone) and HBT (E and F). Values are expressed as the mean ± S.E.M. from 6 to 8 animals and were analyzed using *t*-test. *** $P < 0.001$ compared to SC animals.

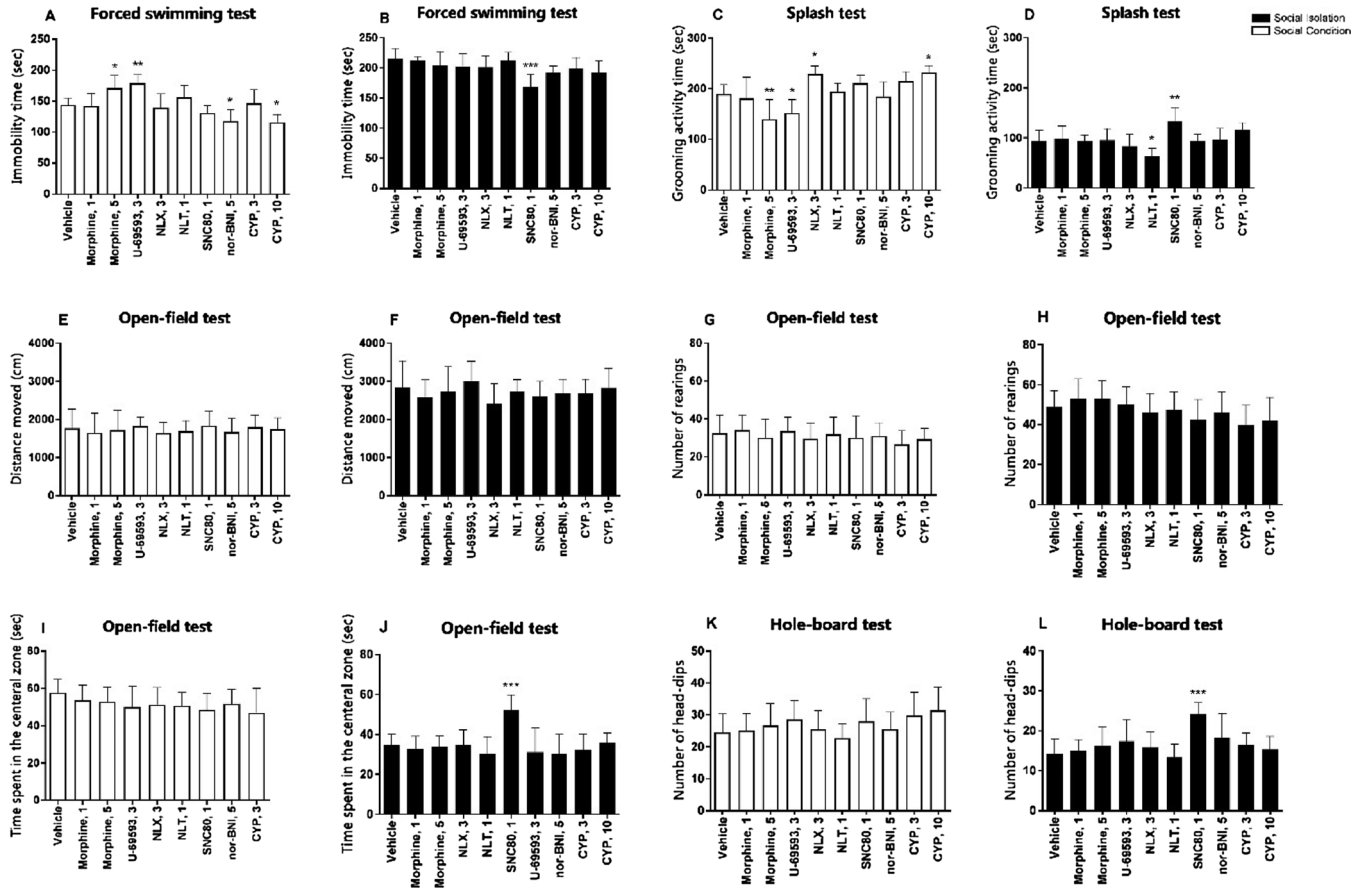

Fig. 2. Effects of morphine (1 and 5 mg/kg), U-69593 (3 mg/kg), naltrindole (NLT, 1 mg/kg), naltrexone (NLX, 3 mg/kg), nor-BNI (5 mg/kg), CYP (3 and 10 mg/kg) and SNC-80 (1 mg/kg) on socially isolated (IC) and normal (SC) animals in some behavioral tasks including FST (A and B), Splash test (C and D), OFT (E, F, G, H, I and J) and HBT (K and L). Values are expressed as the mean ± S.E.M. from 6 to 8 animals and were analyzed using one-way ANOVA followed by Tukey's post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, drug-treated animals from each group were compared to corresponding vehicle-treated animals.

including morphine (1 and 3 mg/kg), U-69893 (3 mg/kg), NLT (1 mg/kg), NLX (3 mg/kg), and SNC80 (1 mg/kg), nor-BNI (5 mg/kg) and CYP (3 and 10 mg/kg) had any effect in the hole-broad results of SC animals compared with vehicle-treatment ($F(9, 70) = 0.4385$, $P > 0.05$, Fig2K). Also, injection of these drugs in IC animals showed no significant effect except for SNC80 ($F(9, 70) = 0.6545$, $P > 0.05$, Fig2L). Post analysis revealed that SNC80 led to anxiolytic-like behavior in the Hole-broad test ($P < 0.001$) by increasing the number of head dips in comparison with vehicle-treated IC animals. Similar results were documented from IC ($F(9, 70) = 1.447$, $P < 0.001$, Fig2I) and SC ($F(8, 63) = 0.9091$, $P > 0.05$, Fig2I) animals in the time spent in the central zone (OFT). Post hoc analysis showed that SNC80 significantly decreased anxiogenic-like behavior in IC mice in comparison with vehicle-treated animals ($P < 0.001$).

3.3. Gene expression of opioid receptors

Our results demonstrated the significant differences in gene expression of opioid receptors in hippocampus ($F(5, 24) = 18.73$, $P < 0.001$, Fig. 3A) and amygdala ($F(5, 24) = 15.01$, $P < 0.001$, Fig. 3B) between IC and SC animals in two-way ANOVA analysis. Tukey's analysis showed that SIS could decrease gene expression of MORs ($P < 0.001$) and KORs ($P < 0.01$) compared to SC animals in hippocampus. Also, data obtained from amygdala showed that MORs ($P < 0.001$) and KORs ($P < 0.05$) gene expression significantly decreased in socially isolated animals. However, there is no significant difference in gene expression of DORs neither in hippocampus nor amygdala between IC and SC animals.

4. Discussion

In the current study, we examined the possible role of the opioid system on anxiety- and depressive- like behaviors in SIS paradigm. Although SIS and opioid system have been investigated previously, the relation between the therapeutic effects of this system on behavioral despair induced by SIS have not been documented well. In this regard, we aimed to assess whether opioid receptors could be considered as a potential target for treatment of the adverse effects of SIS. Also, the gene expression of opioid receptors was evaluated to illuminate the impact of opioid system. Our results showed that administration of delta opioid agonist (SNC80) attenuated immobility time in FST and increased the time spent in central zone and also the numbers of head-dips in OFT and HBT in isolated condition group. In consistency with our behavioral experiments, we found that gene expression of KORs and MORs significantly decreased after social isolation stress in hippocampus and amygdala. This is probably caused by increase in endogenous Kappa and Mu agonist tone. In contrast with KORs and MORs gene expression, DORs gene expression did not alter significantly neither in hippocampus nor amygdala.

A large body of evidence and our past studies showed that SIS could induce anxiety- and depressive-like behavior in some behavioral tasks [10,49] such as the hole-board test (HBT), the open flied test (OFT), forced swimming test (FST) and the splash test. In line with aforementioned studies, we observed that IC animals showed increased immobility time in the FST and decreased grooming activity time in the Splash test. Also, increased moving distance and number of rearings in addition to the decreased time spent in central zone at the OFT revealed

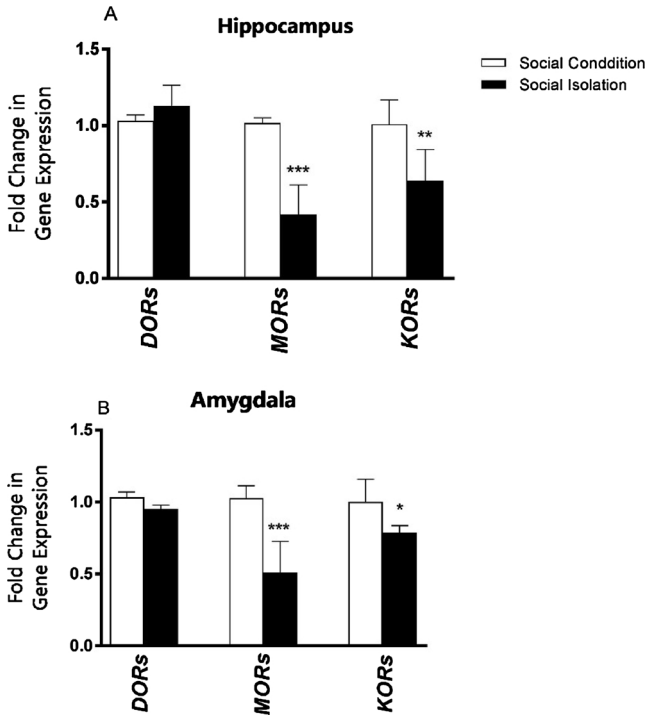

Fig. 3. Effects of social isolation stress on gene expresion of DORs, MORs, and KORs in both hippocampus (A) and amygdala (B). Values are expressed as the mean ± S.E.M. from 4 animals and were analyzed using two-way ANOVA followed by Tukey's post hoc test. $^{**}P < 0.01$, and $^{***}P < 0.001$ were compare to social condition animals.

higher locomotor activity in IC mice. Also consistent with above-mentioned studies, we demonstrated that SIS could induce anxiety-like behavior in the HBT.

The opioid system consists of distributed neurons that produce endogenous opioids including enkephalins, dynorphins and β-endorphins [50]. Endogenous opioids are neurotransmitters and neuromodulators of mu, delta and kappa opioid receptors [51]. Although, the role of opioid system in behavioral functions has been previously investigated, only scarce controversial findings are available in this regard. However, further animal studies showed that this inconsistency could be due to various factors including chronic or acute usage of opioid agonists/antagonists, their dosage, type of targeted receptors (Mu, Kappa, or Delta), species, and etc. In this regard, Poznanski et al. showed that chronic blockade of opioid system by naloxone (non-selective opioid receptor antagonist) could induce anxiety- and depressive-like behavior in rodents [52]. However, other study suggested that acute administration of morphine (selective opioid receptor agonist) could led to increased anxiety- and depressive-like behavior in animals similar to chronic naloxone treatment [53]. Nonetheless, there is little information on the relationship between chronic stressors such as SIS and opioid system. Our results suggest that opioid system might be involved in the adverse effects of SIS. Hence, below we will discuss about the potential associations of each opioid receptor with mood disorders in details.

4.1. Kappa opioid receptor (KORs)

The role of KORs in pathophysiology of depression and anxiety is not fully understood; however, several studies have shown that KOR antagonists possess antidepressant-like effect in behavioral tasks [34,38,54–56]. Past studies revealed that activation of dynorphin/KOR pathway in stress-related brain regions could have led to behavioral disorders [57,58]. It has been shown that increase in the level of dynorphin following stress could reduce releasing of the dopamine [5,59,60]. Previous studies demonstrated that dysregulation of mesolimbic dopaminergic system may associate with depressive- like behavior IC mice [61,62]. Likewise social isolation increases the accumbal dynorphin/KOR function which is associated with alteration in nucleus accumbens dopaminergic signaling pathway [5].

In this study, administration of U-69593 (as a selective KOR agonist) in SC animals led to depressive-like behavior in both the FST (by increasing the immobility time) and the splash test (by decreasing the grooming activity time) but administration of nor-BNI (as a selective KOR antagonist), exerted anti-depressant-like behavior in the FST. Injection of U-69593 and Nor-BNI in SC mice failed to cause significant effect in the hole-board test and the OFT. Interestingly, we observed that nor-BNI did not possessed anti-anxiety and anti-depressive-like behaviors in IC animals.

4.2. Delta opioid receptor (DORs)

It has been demonstrated that activation of DORs increase the release of norepinephrine and thus could mitigate depression and anxiety [63,64]. Animal studies show that deletion of the DOR gene induces anxiety-like behaviors [65]. Likewise, prior reports demonstrate that administration of DOR antagonists increases anxiety-like behaviors [66,67], while DORs agonists reduce the aforementioned behavioral impairments [68,69]. According to previous studies, injection of enkephalinase inhibitors increases the level of enkephalins (as ligands of DOR), leading to antidepressant effects [70]. Broom et al., also revealed that administration of SNC80, a DOR agonist, induces antidepressant-like behavior in the FST [71]. In the present study, administration of SNC80 (a selective DOR agonist) in SC animals did not affect the FST, OFT, splash test, and hole-broad test. However, in IC mice, SNC80 induced anxiolytic- and antidepressant-like effects. Also, injection of DOR antagonist (naltrindole) in SC could not induce any effect in all exprimental tasks. However, administration of naltrindole possessed depressant-like effect in the Splash test (not in the FST).

4.3. Mu opioid receptor (MORs)

MORs has a key role in reward system [59,72]. Studies showed that MORs are involved in the pathophysiology of anxiety and depression [73]. In this regard, it has been demonstrated that deletion of the MORs gene could decrease anxiety- and depressive-like behaviors in animals [74]. Controversially, some studies have indicated that activation of MORs could result in antidepressant- and anxiolytic-like effects [75]. In this study, we used morphine (K_i: 14 for MORs, 538 and 1000 for KORs and DORs [76], respectively) and cyprodime hydrochloride to evaluate the role of MOR on behavioral despair induced by SIS. Administration of morphine exerted depressant-like effect in SC animals at dose of 5 mg/kg in both the FST and the Splash test, while cyprodime hydrochloride at dose of 10 mg /kg induced anti-depressant –like effect in both FST and splash test. However, injection of morphine and CYP could not affect any experimental tests in IC animals.

In the final step, for clarifying the above results, we investigated the gene expression of ORs in the hippocampus and amygdala. In this study, we observed that gene expression of the KORs and MORs but not DORs, significantly decrease following SIS. Taken together these data suggests that opioid receptors are involved in pathophysiology of anxiety- and depressive-like behavior induced by SIS. On the other hand, intact DOR expression in addition to anti-depressant and anxiolytic-like effect of DORs agonist in socially isolated animals suggest that DORs agonist could be used as a new and novel treatment to decrease SIS consequences including anxiety and depression.

Although, evaluating of endogenous opioids could make our hypothesis stronger, in the current study, the aforementioned evaluation has been restricted due to our limitations. In this regard, this manuscript recommends future studies to evaluate the activity of endogenous opioid peptides in socially isolated animals for clarifying the role of

opioid system in behavioral despair induced by SIS.

5. Conclusion

In this study, our results hypothesized that SIS could probably increase endogenous opioids which may interact with MORs and KORs but not DORs. Also, it could be concluded that DORs agonist might be a novel target for treatment of depression and anxiety in SIS. Based on our molecular study, intact DORs and probably its endogenous, in contrast with other opioid receptors, suggested that targeting of DORs could use as a novel treatment of behavioral despair induced by SIS.

Conflict of interests

The authors declare no conflict of interest.

Compliance with ethical standards

The authors declare no conflict of interest. Also, all applicable international and institutional guidelines for the care and use of animals were followed.

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